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Prevalens av atypiska *Leptospira* serovarer i Nya Zeelands betesdjur

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ABSTRACT

The aim of this study was to estimate the overall prevalence and herd/flock seroprevalence of the *Leptospira* serovars Ballum, Copenhageni and Tarassovi and the differences in prevalence between regions in New Zealand. The samples used in the study were 3,878 stored serum samples from beef cattle, deer and sheep. The samples came from 9 different regions and from both the North Island and the South Island. The samples were analysed by MAT (microscopic agglutination test) using the titre cut-off point 1:48. Seroprevalence in beef cattle were 13.7%, 14.7% and 18.0% for Ballum, Copenhageni and Tarassovi, respectively. In deer the corresponding figures were 6.6%, 15.5% and 3.6% and in sheep 10.5%, 16.7% and 14.0%. When a farm was regarded as positive as long as at least one positive animal was found, the prevalence of farms positive for Ballum was 76.0% and 88.4% for Copenhageni and 74.0% for Tarassovi. Two farms had no positive samples for any of the three serovars. The prevalence of the three serovars could also been seen to vary between the different regions, although there was a huge difference in number of samples from the regions. Ballum seemed to have a lower prevalence in the South Island than in the North Island. The origin of these serovars is unknown. Based on earlier findings, however, a working hypothesis for future studies is that domestic livestock may be infected through contact with infected wildlife species. As serovars Tarassovi and Ballum are not included in any of the vaccines registered in New Zealand, this study demonstrated that vaccination cannot fully protect farmers against exposure to *Leptospira*.

SAMMANFATTNING (ABSTRACT IN SWEDISH)

Målet med den här studien var att skatta den generella prevalensen och gårds-/flock prevalensen av *Leptospira* serovarerna Ballum, Copenhageni och Tarassovi och skillnaderna i prevalens mellan olika regioner i Nya Zeeland. Proverna som användes i studien bestod av 3,878 lagrade serum prover från nöt (köttdjur), hjort och får. Proverna kom från nio olika regioner och från både nordön och sydön. Proverna analyserades med hjälp av MAT (mikroskopiskt agglutinations test) och tröskelvärdet (titre cut-off point) som användes var 1:48. Prevalensen hos nöt var 13,7 %, 14,7 % och 18,0 % för respektive Ballum, Copenhageni och Tarassovi. Hos hjort var prevalensen 6,6 %, 15,5 % och 3,6 % och hos får låg den på 10,5 %, 16,7 % och 14,0 %. Med antagandet att en gård var positiv så länge det fanns minst ett positivt djur på gården så blev gårdsprevalensen för Ballum 76,0 % samt 88,4 % för Copenhageni och 74,0 % för Tarassovi. Två gårdar hade inga positiva prover för någon av de tre serovarerna. Prevalensen för dessa tre serovarer varierade mellan regionerna, dock så var det en stor skillnad i antalet prover från de olika regionerna. Ballum verkade ha en lägre prevalens på sydön än på nordön. Eftersom serovarerna Tarassovi och Ballum inte är inkluderad i något av vaccinerna som är registrerade i Nya Zeeland, så visar denna studie att vaccinering ej kan skydda bönderna fullt ut mot exponering av *Leptospira*. Var dessa serovarer har sitt ursprung ifrån är okänt, men baserat på tidigare studier kan man inför framtida studier arbeta efter hypotesen att domesticerade produktionsdjur kanske infekteras via kontakt med vilda djur som är infekterade.

CONTENTS

List of abbreviations

MAT	Microscopic Agglutination Test
EMJH	Ellinghausen-McCullough-Johnson-Harris media
µl	Microliter
ml	Milliliter
PCR	Polymerase Chain Reaction
qPCR	Real-time Quantitative Polymerase Chain Reaction

1. INTRODUCTION

1.1. Background

Leptospirosis is a zoonotic infectious disease of global importance that can cause serious consequences to the health of both animals and humans (Bharti et al., 2003). It occurs in both low and high income countries. It is a more common disease in the tropics because the conditions for transmission are favourable in the more humid type of climate that occurs in the tropics; however it often gets neglected due to the amount of other zoonotic diseases occurring in the tropics (Bharti et al., 2003). In New Zealand the clinical disease leptospirosis is relatively well-known (Subharat et al., 2012). Leptospirosis is caused by motile leptospires which are bacteria (obligate aerobic spirochetes, with a spiral shaped form) of the genus *Leptospira* spp. *Leptospira* is divided into different species, including both pathogenic and non-pathogenic members (Dreyfus et al., 2013). Based on outer-membrane antigen structure, species are subdivided into serovars of which a large number is known to be pathogenic for mammals. As many as 193 serovars have been catalogued only within the specie *L. interrogans* (Kmety & Dikken, 1993: see Adler, 2015 p.12), although only six of them are known to be endemic in New Zealand (Dreyfus et al., 2013). One of which is serovar Pomona. The specie *L. borgpetersenii* consists of, among others: serovar Tarassovi, serovar Hardjobovis, serovar Ballum and serovar Copenhageni. All these serovars occur in New Zealand among animals and humans (Heuer et al. 2008).

Mammals (both domestic and wild), reptiles and amphibians all serve as maintenance hosts for the genus *Leptospira* (Plank & Dean, 2000). The two main serovars in New Zealand livestock are *L. interrogans* serovar Hardjobovis and serovar Pomona. Serovar Hardjobovis which is maintained in cattle is distributed almost globally around the world. However there are countries where it seldom occurs or is completely absent, amongst those countries are the Scandinavian ones (Adler et al., 2014). For example in Sweden there have not been any findings of serovar Hardjobovis and in a study the prevalence of leptospirosis was noted as low as 1% in dairy cows. The serovar that was noted was a serovar similar to serovar Sejroe called strain Mouse 2A (Lindahl et al. 2011).

Regarding hosts for different *Leptospira* serovars there is a difference between maintenance hosts and accidental hosts. The pathogenicity is assumed to be lower in maintenance hosts than in accidental hosts, but they are regarded as being almost equally infectious (Heuer et al., 2012). In New Zealand serovar Hardjobovis also seems to be adapted to deer, apart from cattle, whereas sheep are considered to be only sporadically infected. However, there is evidence of Hardjobovis becoming adapted to sheep as well (Dreyfus et al., 2013). *L. borgpetersenii* serovar Tarassovi, Ballum and Copenhageni have other hosts that they are adapted to. Tarassovi is adapted to pigs, while Ballum and Copenhageni are adapted to rodents (Dreyfus et al., 2013) and Ballum is

adapted also to hedgehogs. Both Ballum and Copenhageni are adapted to the house mouse and the ship rat. It is unusual for one single serovar to be adapted to as many as three different species (Hathaway, 1981).

Many farms in New Zealand have multi-species pastoral systems that use co-grazing, where transmission of pathogens could be possible. Leptospires can be found in freshwater and are excreted in the urine of infected animals, since it mainly resides in the kidneys of the animals. Carrier animals can excrete it in the urine during months and even years (Dreyfus et al., 2013). The animals can be acutely infected or chronic and the symptoms may vary, animals can also be asymptomatic. In cattle clinical symptoms such as pyrexia, haemolytic anemia and hemoglobinuria can occur. Even meningitis is possible and death. If infected by Hardjobovis the symptoms often remain subclinical except in lactating cows where agalactia may occur. Chronic leptospirosis can cause birthing problems in cattle, such as stillbirth and abortion (Ellis, 2015). Hardjobovis have been seen to cause losses between birth and weaning in red deer in New Zealand (Ellis, 2015). The acute symptoms in sheep are similar to those noted in cattle, but for the fact that they are mostly seen in lambs (Ellis, 2015). It can infect animals and people through damaged skin and mucosa; it then multiplies in different organs like the kidneys and liver, but also in the central nervous system (Johnson, 1996). One of the most common infection routes for humans is through contact with soil or water that have been contaminated with urine and through contact with animal tissue. Rat bites are another infection route as well (Plank & Dean, 2000).

1.2. Vaccines

There are vaccines against *Leptospira* and animals do get vaccinated against *Leptospira* in New Zealand, although not all farmers choose to vaccinate. Presently there are nine leptospirosis vaccines for cattle, three for deer and two for sheep, available in New Zealand. These include vaccine against Hardjobovis and Pomona, which are the two main serovars present in New Zealand. There are however some vaccines that also include the serovar Copenhageni, but none that includes Ballum and Tarassovi. If the animal has not been infected prior to the vaccination, the vaccines should prevent urinary shedding, however more studies need to be done in order to see if that actually works (Dreyfus et al., 2013). The vaccine is also beneficial in the way that it reduces the clinical disease that are affecting the health of the animal (Benschop et al., 2012)

1.3. Leptospirosis in humans

Ballum, Copenhageni and Tarassovi are all serovars occurring in humans (Heuer et al., 2008). The people at highest risk of getting *Leptospira* are especially farmers, meat workers and veterinarians, why livestock is seen to be an important infection route for human leptospirosis

(Dreyfus et al., 2013). Leptospirosis in humans can cause severe illness, however in New Zealand it is rarely fatal. A problem is that it is probably a lot of cases that remains undiagnosed since the symptoms can be very similar to those caused by influenza (Bharti et al., 2003). Sadly there is also a lack of knowledge and awareness about the disease among the common population (Dreyfus et al., 2013). That is one of the reasons why it is important to learn more about *Leptospira* and its different serovars, prevalence in animals and the possibility of transmissions to humans as well.

1.4. Aim

The primary aim of this study was to estimate the overall prevalence of Ballum, Copenhageni and Tarassovi in New Zealand, using serum samples of sheep, cattle and deer. We also aimed to determine the herd/flock seroprevalence of the three serovars and to investigate if the prevalence was different in the different regions in New Zealand.

2. LITERATURE REVIEW

2.1. How to detect *Leptospira*

There is no possibility to diagnose a *Leptospira* infection just through clinical findings. Cattle suffering from acute leptospirosis show post-mortem findings such as kidney lesions and there may also be liver lesions, although not quite as clear as the kidney lesions (Faine, 1982). In sheep and goats enlarged kidneys with petechial hemorrhages may be observed and these animals can also show a variable degree of icterus post-mortem (Faine, 1982).

There are different laboratory diagnostics of leptospires. There are tests based on detecting the DNA of the organism by molecular methods (Fang et al., 2014). Today PCR is also a common test to use and it is considered reliable and rapid when it comes to diagnose leptospirosis. The qPCR is time-saving compared to the conventional PCR and at the same time it is less likely that contamination of the test occurs (Fang et al., 2014).

Leptospires can also be isolated by cultures or also through animal inoculation procedures. Leptospires can be isolated from the blood and also from the tissues where they are present, such as the liver, spleen, kidneys, brain and also from aborted fetuses. There is also serological testing such as the microscopic agglutination test (MAT) which is the most frequently used method to diagnose leptospirosis (Faine, 1982). The MAT is also considered to be the reference test for serological diagnosis of leptospirosis. However the MAT cannot differentiate between naturally

infected animals and vaccinated animals. Another problem is that cross-reactivity can occur if the serovars are closely related (Fang et al., 2014).

The specificity for the MAT has in earlier studies been shown to be very high, varying between 96.4% and 100%. The sensitivity has also been shown to be high, although it has a big range from 34.0% to 100% (Hea, 2014). In a study evaluating four serological tests for diagnosis of leptospirosis MAT was found to have a specificity of 97.3% and a sensitivity of 98.2%, other studies have shown similar results (Bajani et al., 2003). However the specificity of MAT can vary between different countries, depending on what serovars there is in the country. If there is a lot of serovars which are similar to each other the specificity of the test will be lower, and conversely if there are fewer serovars that are not similar to each other the specificity would be higher. Also the sensitivity depends on which serovar is being tested (Collins-Emerson, personal communication). The sensitivity and specificity of PCR and MAT were tested with Bayesian estimates in a study conducted in 2014. The sensitivity and specificity were tested in beef cattle and sheep using the serovars Hardjobovis and Pomona. The sensitivity of MAT for detection of leptospirosis infection was then found to be 84.0% in sheep and 83.0% in beef cattle, while the specificity was 73.0% and 44.0% in sheep and beef cattle, respectively (Hea, 2014). The sensitivity has not been calculated for Ballum, Copenhageni and Tarassovi in New Zealand.

In the event of diagnosing leptospirosis, PCR has shown similar sensitivity as serological methods and has been shown to be far more sensitive than DNA-based techniques in a study. The specificity however depends on multiple factors through the testing (Van Eys et al., 1989). In the study by Hea (2014) the sensitivity of PCR was 65.0% and 53.0% in sheep and beef cattle, respectively, while the specificity was 97.0% and 96.0%. The sensitivity and specificity of qPCR depends on the onset of infection, with a higher sensitivity in the beginning of the infection. In a study the sensitivity could be seen to be 100% in the beginning of the infection and then dropping to 69.0% after 5-10 days. The specificity was around 100% (Ahmed et al., 2009).

2.2. Prevalence of *Leptospira* serovars in animals

An investigation of the occurrence of Hardjobovis and Pomona in New Zealand has been done; blood was sampled from 7,661 animals throughout New Zealand from different regions and different species (deer, cattle and sheep). The result from the study showed that 43% of the sheep samples were positive against Hardjobovis, whereas 14% were positive against Pomona and 50% were tested positive for both serovars. For cattle the result was 50% against Hardjobovis, 25% against Pomona and 58% against either of the serovars. The deer samples resulted in 26% seropositive against Hardjobovis, 11% against Pomona and 34% against either of the serovars

(Dreyfus et al., 2013). There was no evidence that the seroprevalence in deer co-grazing with sheep or beef cattle had any statistically significant associations. No significant effect of co-grazing could be seen in sheep or beef cattle either (Dreyfus et al., 2013).

In a study carried out in Papua New Guinea 1,300 samples were collected from female cattle and tested against 21 different serovars through MAT (Wai'in et al., 2006). Among the ones that occurred most were Hardjobovis, Pomona and Tarassovi. Hardjobovis was the most dominant one with a seroprevalence of 53.7%, while Tarassovi was noted with a seroprevalence of 15.5% and Pomona had a seroprevalence of 8.4%. The two other serovars which had a high seroprevalence (Szwajizak and Medanensis) are believed to have cross reactivity with serovar Hardjobovis. Those farms that showed a high seroprevalence of Tarassovi had a close association with pigs (Wai'in et al., 2006). Also in Australia a high seroprevalence of Tarassovi has been noted. In a study of 68 beef herds in Queensland Tarassovi occurred among the most commonly detected serovars with a seroprevalence of 13.9%. Also Hardjobovis and Pomona were among those most commonly detected (Heuer, 2006). However, the serovars differ worldwide and an entirely different serovar can be the most common one in another country. Because of this it can be hard to make comparisons between countries in occurrence of serovars.

Different studies in New Zealand have shown different seroprevalence of Copenhageni throughout the years. In a survey conducted in 1988 among deer herds the overall prevalence was estimated to be 16.6% (Flint et al., 1988). A herd-level prevalence in deer herds from the lower part of the north island was 11.3% in a study published in 1998 (Wilson et al., 1998). In a study performed in 2010 no evidence of herd-level infection with Copenhageni was found (Ayanegui-Alcérecca et al., 2010). In the study performed 1998 Copenhageni was noted to have a lower prevalence than Pomona and Hardjobovis in the investigated area. However they also detected Ballum and Tarassovi as well, but during this time the significance of these serovars were unknown and more research needed to be done in order to get to know their possible significance (Wilson et al., 1998).

2.3. Prevalence of *Leptospira* serovars in humans

New Zealand had an incidence of 2.4 cases per 100,000 individuals in 2012 and an incidence of 1.3 in 2013 (Anon., 2013; 2014). Since there is a high prevalence of *Leptospira* serovars occurring amongst livestock in New Zealand, surveys have been done to investigate the risk for, among others, meat workers and meat inspectors. The seroprevalence for Hardjobovis and Pomona noted in a study from 2009 was 9.5% among meat workers in a sheep abattoir (Benschop et al., 2009). What also has been shown is that meat workers are at a constant risk of

exposure of *Leptospira* (Dreyfus et al., 2014). In the study samples were collected from meat workers working in different places of the slaughter line to investigate where the risk for a new infection was highest. The highest risk could in sheep abattoirs be seen to be in the beginning of the slaughter line. In the beginning of the slaughter line there is probably a higher risk to get in contact with contaminated urine and internal organs (Dreyfus et al., 2014).

In a study conducted among New Zealand veterinarians the purpose was to investigate the seroprevalence of the serovars Copenhageni, Ballum, Tarassovi, Hardjobovis and Pomona (Sanhueza et al., 2015). The seroprevalence for all five serovars together was noted as 5.1% although no veterinarian was noted as seropositive for Tarassovi. A seroprevalence of 0.4% was noted for both Ballum and Copenhageni and a seroprevalence of 2.2% for Pomona and 2.5% for Hardjobovis (Sanhueza et al., 2015). However no direct association was found between the species of animals the veterinarians were working with and infecting serovar and the seroprevalence was noted half as high as in abattoir workers (Sanhueza et al., 2015). Of 88 notified human cases in New Zealand during 2006, 77 had the actual serovar recorded. Of these there were 36 cases of Hardjobovis, 18 cases of Pomona, 16 cases of Ballum, 6 cases of Tarassovi and 1 case of Copenhageni (Heuer et al., 2008).

Another study which was undertaken in Malaysia investigated the seroprevalence of leptospirosis in humans. A set of 198 blood samples were collected from different humans and 35.9% were positive for leptospirosis using the MAT. Seventeen different *Leptospira* serovars were detected, the most common being Sarawak (Thayaparan et al., 2015). In Australia the highest incidence of leptospirosis has been noted to be in Queensland and Northern territory during 2013, with 1.4 and 1.7 cases per 100, 000 respectively (de Kluyver et al., 2015). Most countries do not keep a yearly record of the incidence of leptospirosis. However it seems to be an important growing public health problem and there are increasing amount of reports of outbreaks, especially from Africa and the Middle East (Abela-Ridder et al., 2010). Climate change, such as heavier rainfall and flooding, seem to give rise to outbreaks of leptospirosis in humans (Abela-Ridder et al., 2010).

3. MATERIAL AND METHODS

3.1. Collecting and preparing samples

The material for this study consisted of 3,878 stored serum samples, which have been randomly selected out from a collection of 7,661 serum samples. These samples were collected by different veterinarians throughout New Zealand during 2009-2010. Details of the collection can be found in Dreyfus et al. (2013). Briefly, the samples were collected from both the north and the south

island from eight different regions; Waikato, Wairarapa, Hawke's Bay, Manawatu-Wanaganui, Taranaki, Marlborough, Canterbury and Southland. Farms were selected by stratified sampling from a sampling frame of 1,914 farms that had responded to a survey mailed out to about 8,500 farms with questions about *Leptospira* and Johne's disease. The single stratification criterion was species composition, i.e. either sheep, deer and beef cattle in isolation, or mixed species farms where species were mostly co-grazed together. The farms had to have a minimum number of animals: 40 deer, 400 sheep and/or 40 beef cattle. Samples were collected from mixed age ewes, beef cows and hinds and included both vaccinated and unvaccinated animals.

The collection of samples often consisted of 20 samples from each animal species from each farm and because a lot of NZ farmers keep both deer, cattle and sheep there were up to 60 samples from some farms. In conjunction with collecting the samples the serum was separated from blood by centrifugation and stored in -80 degrees. All sera had earlier been tested for serovars Hardjobovis and Pomona, while this study tested for Copenhageni, Tarassovi and Ballum.

3.2. Setting up master plates

In order to do the MAT, the serum samples had to be set up in master plates. A master plate is a plate that consists of 96 wells and is prepared with one sample in each well. During the study a total of 42 plates were prepared (not all plates had samples in each well, due to gaps that were left between samples if numbers were uneven). The wells were set up with 30 µl of serum sample in each well and 150 µl of sterile saline solution. The master plates were labelled with number and date of preparation and then stored in the -20 degree freezer in wait for the MAT. Along with the preparation of the plates, protocols were written in order to know which samples were in which plates.

3.3. Preparation of cultures

The cultures that were used for the MATs were prepared with 80ml of liquid leptospiral culture medium (EMJH) in which 1ml of the chosen strain was added. These cultures were then allowed to grow during 2-3 days in a 27-degree cabinet on a shaker. The culture had to be checked under dark-field microscope before setting up the plates. If the culture had grown too old and self-agglutinated too much it was not possible to use that culture for the MATs. If there was only a small part of agglutination in the culture, it was possible to dilute the culture with some millilitres of sterile saline solution, and also if the culture was regarded as too dense. After diluting it with sterile saline solution it got shaken (to get rid of the agglutination) and then it was ready to be used.

3.4. Microscopic agglutination test

The procedure for the MAT is as follows. The master plates that were planned to be used during one day were taken out of the freezer to defrost. For each master plate 8 plates were needed for the dilutions. Figure 1 shows how the plates were set up. These plates were filled with 25 µl saline in each well (one plate contains 96 wells). To each testing of a serovar a plate had to be set up as a control where 25 µl of sterile saline solution were put in the first two rows of that plate. In the second row 25 µl extra saline was put and diluted with 2-fold serial dilution through eight wells. In the first row, which functioned as the positive control, the antiserum was put for the requested serovar that would be done that day. Twenty-five µl of antiserum was placed in the first well and prepared in the same manner as the negative control. The other plates had 25µl of serum diluted down eight wells with 2-fold serial dilution, from 1:24 to 1:3072. The plates were then set up with 25µl of the desired culture in each well and the control plate was set up in the same way.

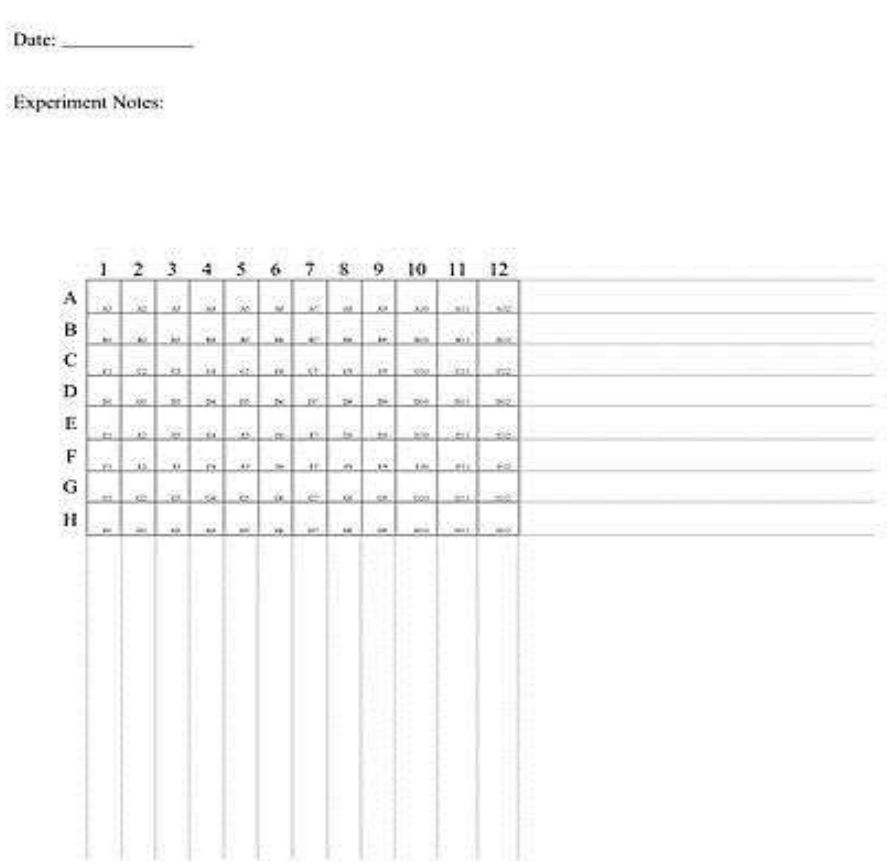

Figure 1. *The antiserum was placed in column one and the negative control in column 2 and diluted with 2-fold serial dilution from 1:24 to 1:3072. The sample plates had 12 different samples in each plate on sample in each column (1-12) (© Wikimedia Commons, images available under a Creative Commons Attribution-Noncommercial license*).

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The plates were then incubated in a 37 degree room for approximately 2 hours. After that the plates were retrieved and ready to be read. The samples were placed on microscope slides (four samples with the eight dilutions of each sample on each slide) and looked at under a dark-field microscope to examine if there was agglutination and also to get the titres determined. The end-point was defined to be the highest dilution of serum where 50.0% agglutination occurred. The control was looked at first, in order to see what the culture looked like and determine the end-point. If it looked alright, meaning that the positive should contain agglutination and the negative should not, it was ok to go ahead and read the samples.

The serum samples were tested against live antigen of serovars Ballum, Tarassovi and Copenhageni.

3.4. Reading MAT

The titre cut-off which was used throughout the study was 1:48 and that titre answer the question whether the animal has been exposed for the serovar. If a sample was to be regarded as positive there should have been agglutination in at least the 1:48 dilution. If there was not any agglutination in the sample or just agglutination in the 1:24 dilution it was regarded as negative. Once there was a sample with agglutination in it, the end-point was determined, i.e. the last dilution with agglutination. The results were written down and stored in folders and later on added to a database.

The preparation of the master plates took two weeks to finish afterwards followed two days of training in reading MATs. The MATs were read during a period of 6.5 weeks. Four master plates were tested each day on a flowing schedule of testing Ballum, Tarassovi and Copenhageni.

3.5. Statistical analysis

Serological test results were entered into an Access© database and analysed using Microsoft Excel©. The data entered in the excel sheet contained information about survey ID, sample ID, species, island, region and the result of the tested serovars Tarassovi, Ballum and Copenhageni. Also the results from the previously tested serovars, Hardjobovis and Pomona, were available in the database. Prevalence's of the serovars according to species, regions and farm/herd level, with exact binomial confidence intervals, were calculated using the calculation program at the website epitools.ausvet.com.au. A farm was regarded as positive as long as it had one positive sample.

The prevalence for the different serovars was tested for cross-reactivity between each other, by applying a Kappa-test. The strength of agreement in a Kappa-test is considered as poor if it is below 0.2, fair if it is between 0.2-0.4, moderate if it is between 0.4-0.6, good if it is between 0.6-0.8 and very good if it is above 0.8

4. RESULTS

Table 1 shows the number of different farms used in the study and how many farms there were with each type of animal constellation.

Table 1. *Number of different farms in the study with different types of animal constellation (n=146)*

Type of farm	Beef cattle	Deer	Sheep	Beef cattle - Deer	Beef cattle - Sheep	Deer - Sheep	Beef cattle - Deer - Sheep
Number of farms	27	34	38	3	20	20	4

The overall seroprevalence in beef cattle was 13.7%, 14.7% and 18.0%, for Ballum, Copenhageni and Tarassovi respectively. In deer corresponding figures was 6.6%, 15.5% and 3.6%. While in sheep they were 10.5%, 16.7% and 14.0%.

Figure 2 shows the distribution of samples according to the Ballum titre cut off. Samples with zero were completely negative. There are a lot of samples ranging between 0 and 1:48, but only a few samples with an end-point higher than 1:96. Similar patterns were seen also for Copenhageni and Tarassovi.

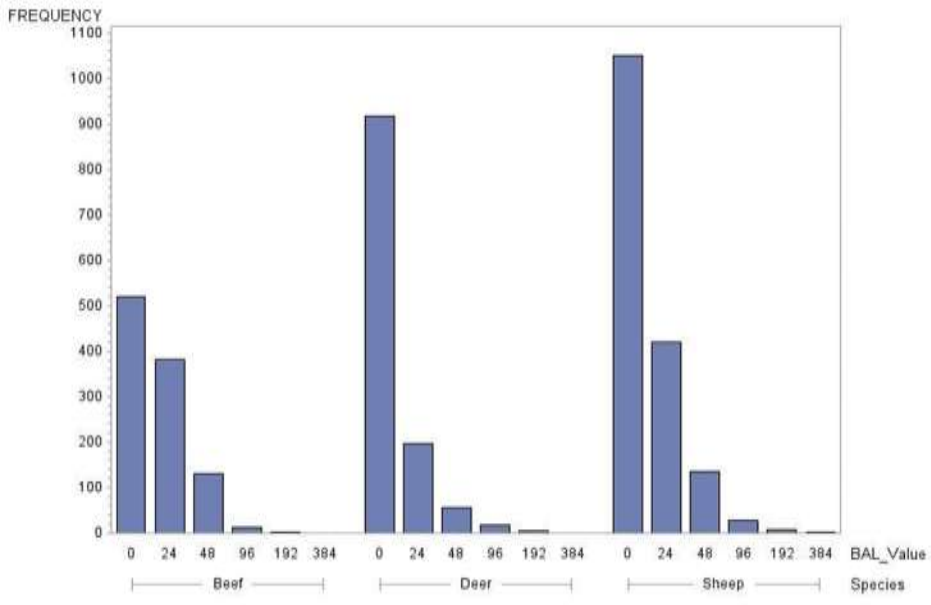

Figure 2. *The distribution of samples according to the Ballum titre cut off. The x-axis shows titre end-points for the samples from beef cattle, deer and sheep.*

Table 2 shows the seroprevalence for the serovars in the different species.

Table 2. *Number of samples positive, and seroprevalence (95% Confidence Interval), for three Leptospira serovars in different species*

	Ballum		Copenhageni		Tarassovi	
	n	%	n	%	n	%
Beef cattle, n=1043	143	13.7 (11.7-16.0)	131	12.6 (10.6-14.7)	188	18.0 (15.7-20.5)
Deer, n=1193	79	6.6 (5.3-8.2)	185	15.5 (13.5-17.7)	43	3.6 2.7-4.8)
Sheep, n=1642	172	10.5 (9.0-12.1)	274	16.7 (14.9-18.6)	230	14.0 (12.4-15.8)

Figure 3 shows the seroprevalence in different regions of New Zealand. Some regions in the maps do not have a number, which means that no samples were collected from these regions.

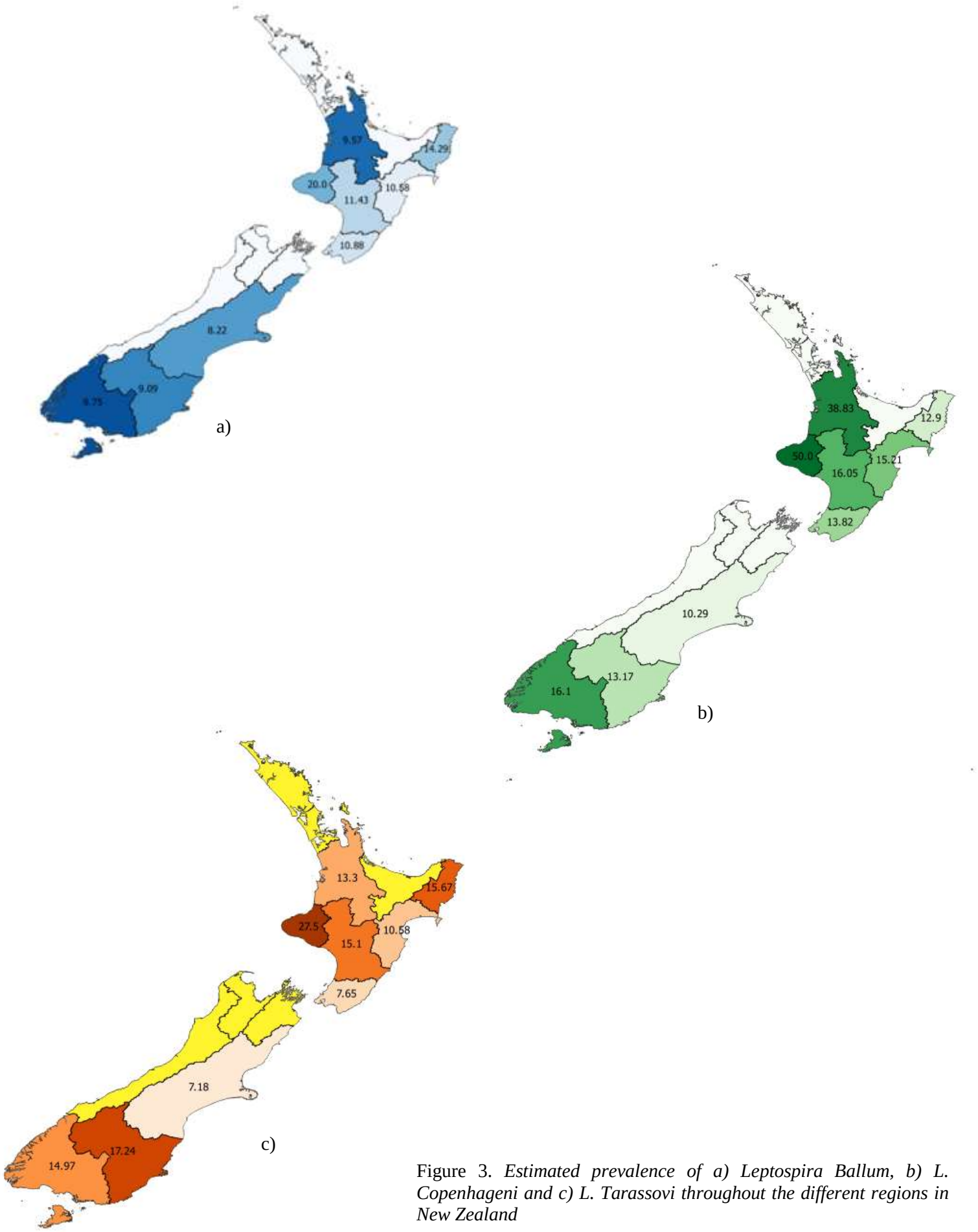

Figure 3. *Estimated prevalence of a) Leptospira Ballum, b) L. Copenhageni and c) L. Tarassovi throughout the different regions in New Zealand*

Table 3. *Estimated prevalence, with 95% confidence intervals of Leptospira serovars Ballum, Copenhageni and Tarassovi, throughout the different regions in New Zealand*

Region	Number of samples	Ballum %		Copenhageni %		Tarassovi %	
Canterbury	1059	8.2	6.6-10.0	10.3	8.5-12.3	7.2	5.7-8.9
Southland	441	9.8	7.1-12.9	16.1	12.8-19.9	15.0	11.8-18.6
Otago	319	9.1	6.2-12.8	13.2	9.7-17.4	17.2	13.3-21.8
Manawatu – Wanaganui	735	11.4	9.2-14.0	16.1	13.5-18.9	15.1	12.6-17.9
Hawkes bay	539	10.6	8.1-13.5	15.2	12.6-18.5	10.6	8.1-13.5
East coast	217	14.3	9.9-19.7	12.9	8.7-18.1	15.7	11.1-21.2
Waikato	188	9.6	5.8-14.7	38.8	31.8-46.2	13.3	8.8-19.0
Wairarapa	340	10.9	7.6-14.2	13.8	10.3-18.0	7.7	8.1-11.0
Taranaki	40	20.0	9.1-35.6	50.0	33.8-66.2	27.5	14.6-43.9

Figure 4-6 show the distribution of within-herd seroprevalence for the different serovars and the different species. There were two farms that were completely free from Ballum, Copenhageni and Tarassovi. Both of these farms were deer farms, one from the Canterbury region and one from the Wairarapa region. Without taking species into account the prevalence of farm positives for Ballum was 76.0% and 88.4% for Copenhageni and 74.0% for Tarassovi.

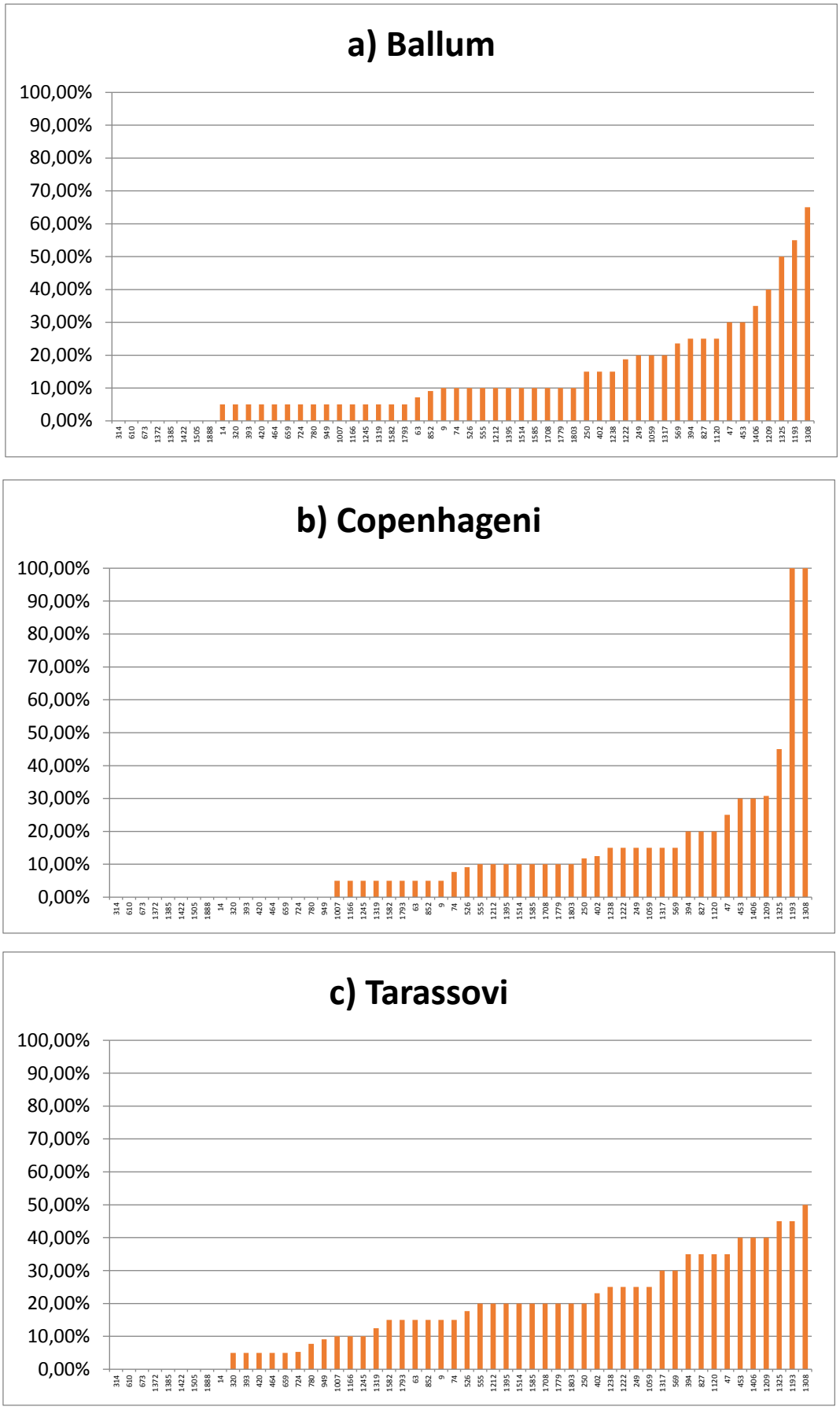

Figure 4. *Within-herd prevalence of three Leptospira serovars sampled in beef cattle farms across New Zealand.*

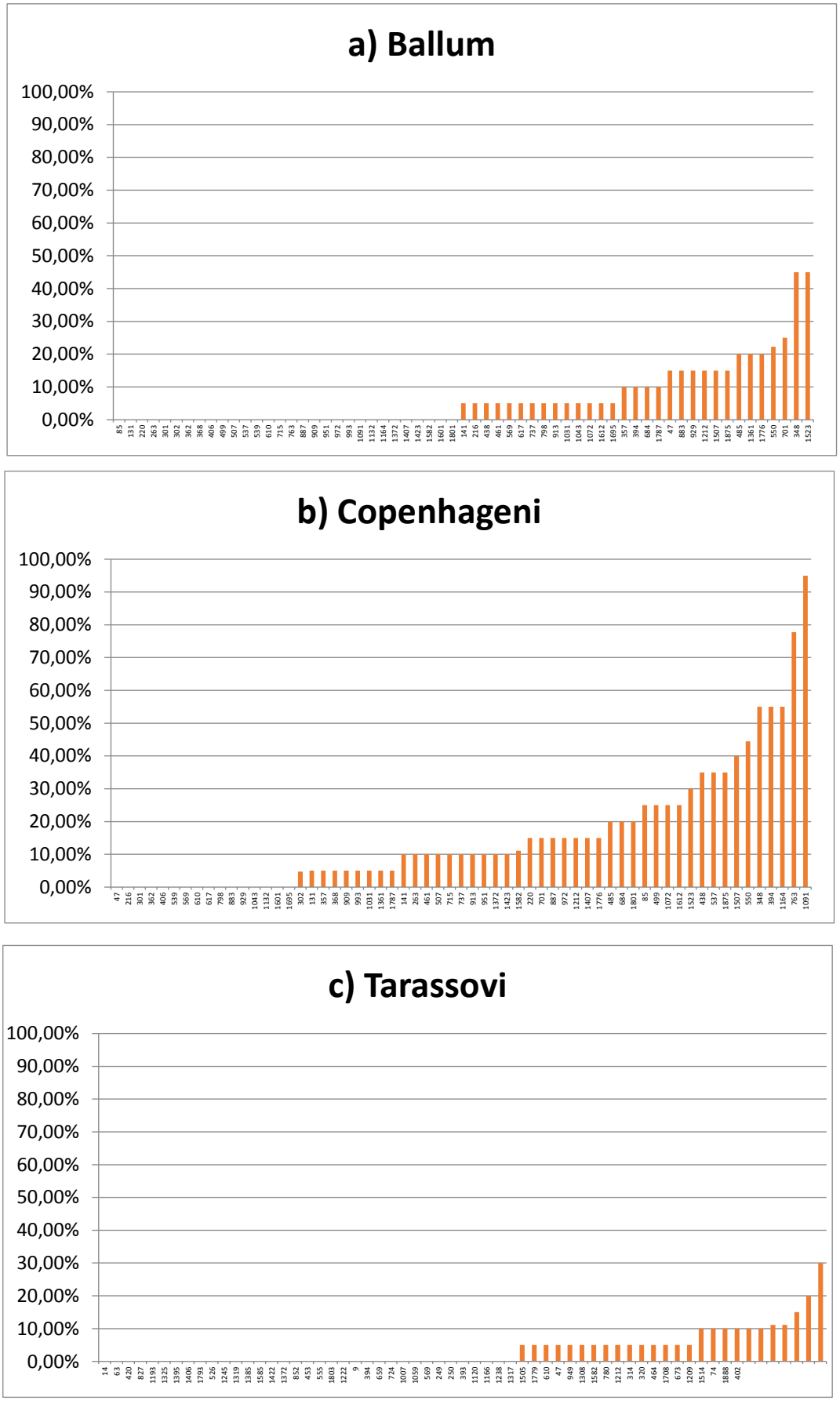

Figure 5. *Within-herd prevalence of three Leptospira serovars sampled in deer farms across New Zealand.*

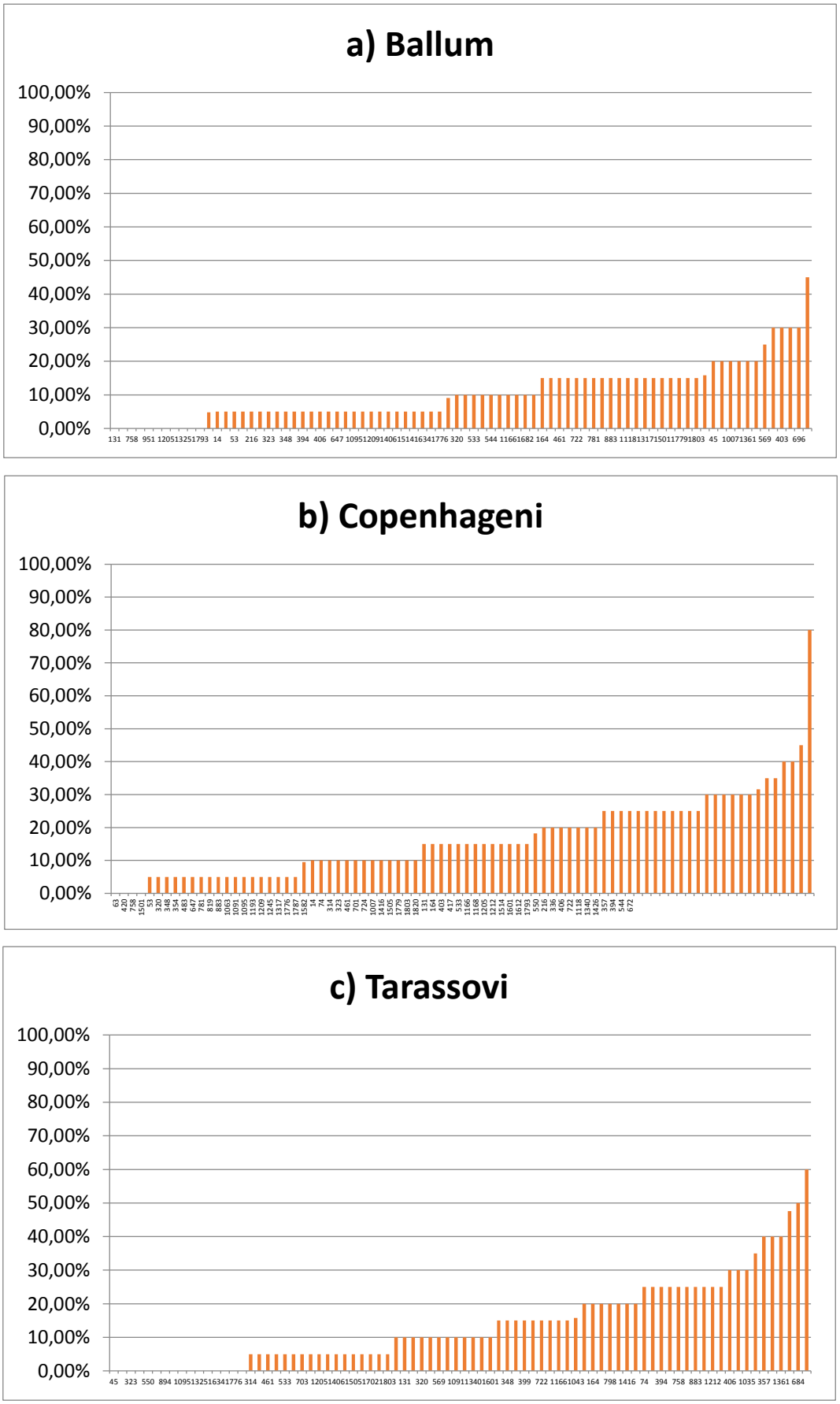

Figure 6. *Within-herd prevalence of three Leptospira serovars sampled in sheep farms across New Zealand.*

As can be seen in table 4 all of the agreements were stated as poor when using the Kappa-test, which means that no significance in agreement was found.

Table 4. *Kappa values between the different serovars. The results were also tested against Hardjobovis and Pomona to investigate possible cross-reactivity. Values below 0.2 are regarded as poor agreement*

Serovar	Ballum	Copenhageni	Tarassovi	Hardjobovis	Pomona
Ballum	-	0.097	0.119	0.06	0.006
Copenhageni	0.097	-	0.05	0.046	0.182
Tarassovi	0.119	0.05	-	0.078	0.072

5. DISCUSSION

The seroprevalence of Copenhageni was 14.7%, 15.5% and 16.7% in beef cattle, deer and sheep, respectively. A similar prevalence was observed in deer in a study conducted in 1988 (Flint et al., 1988), although in a study from 2010 the seroprevalence was only 1.2% (Ayanegui-Alcérreca et al., 2010). The animals in that study consisted of 2,016 deer from 111 herds throughout New Zealand and unlike the samples used in this study the authors selected farms not using a leptospiral vaccine (Ayanegui-Alcérreca et al., 2010). This could explain at least part of the difference in the seroprevalence, because the samples used in our study came from animals that could possibly have been vaccinated with a leptospiral vaccine. The MAT cannot differentiate between a vaccinated animal and an animal that have been exposed to the real serovar. There are not many studies done on sheep and cattle when it comes to the serovar Copenhageni in New Zealand. A study from 1982 showed a seroprevalence of Copenhageni at 2.3% in sheep, but also in this study the samples were from unvaccinated animals (Blackmore et al., 1982). No recent studies that investigate the seroprevalence of Copenhageni in beef cattle and sheep have been found. During 2006 only one human case with the serovar Copenhageni was notified (Heuer et al., 2008)

The seroprevalence of Ballum that was 13.7%, 6.6% and 10.5% in beef cattle, deer and sheep, respectively, was surprisingly high. In a study that was carried out in 1982 (Blackmore et al., 1982) Ballum was noted with a seroprevalence at 2.7% in sheep. The serovar Ballum was observed in a study of deer farms conducted during 1998 (Wilson et al., 1998). However more studies need to be done to know the importance of these findings. To the author's knowledge, this is the first study to detect serovar Ballum in beef cattle in New Zealand. Of the 88 notified

leptospirosis human cases in New Zealand during 2006 16 were caused by the serovar Ballum (Heuer et al., 2008). This shows that Ballum is one of the more frequently occurring serovars in humans in New Zealand causing clinical disease in humans, but the origin of the serovar in these cases is not known.

The seroprevalence of Tarassovi that was 18.0%, 3.6% and 14.0% in beef cattle, deer and sheep, respectively, was also higher than expected. The seroprevalence has earlier been found at 2.6% in sheep (Blackmore et al. 1982) and has been noted in a study conducted on deer farms, although it was not investigated further (Wilson et al., 1998). Also here there is a lack of publications about Tarassovi in cattle. However in both Papa New Guinea (PNG) and Australia, Tarassovi was reported to be at similar levels in cattle as in our study: in PNG serovar Tarassovi was noted with a seroprevalence of 15.5% (Wai'in et al., 2006). The relatively high prevalence of Tarassovi in PNG might have been attributable to contact with pigs. As the pig is a maintenance host for serovar Tarassovi, transmission is likely to occur. In Queensland in Australia, Tarassovi was one of the most detected serovars in cattle with a seroprevalence of 13.9% (Heuer, 2006). This at least shows that Tarassovi is occurring in similar amount in nearby countries. However the yearly notified cases of Tarassovi in humans in New Zealand are low and in 2006 there were only 6 cases (Heuer et al., 2008), but there may be many more that were misdiagnosed or undiagnosed. In a seroprevalence study in 2015 of different serovars in veterinarians in New Zealand (Sanhueza et al., 2015) none of the 297 veterinarians were diagnosed with the serovar Tarassovi. This serovar may therefore have little involvement in clinical leptospirosis of veterinarians.

The seroprevalence of Ballum and Tarassovi in deer were much lower than in beef cattle and sheep, whereas the seroprevalence of Copenhageni showed no differences between species. This may to some extent be explained by the fact that the samples also came from farms that had vaccinated animals against Copenhageni. With respect to Ballum and Tarassovi however, interaction with wild living species may play a role with which deer are likely to interact in different ways resulting in lower contact rates.

The repeated occurrence of Ballum, Copenhageni and Tarassovi in notified human cases seems to indicate that humans are likely to get infected when in contact with beef cattle, deer and sheep. There are no vaccines available in New Zealand today against serovar Ballum and Tarassovi for animals. For humans this poses a risk which is difficult to manage if animals shed these *Leptospira* types as frequently as indicated in this study. Especially meat workers are at risk, because they get in close contact with the internal organs, blood and urine during processing on a daily basis throughout the year. In the study by Dreyfus (2014) about seroprevalence in abattoir

workers, only serovar Hardjobovis and Pomona were investigated, but with the current results at hand it is imperative to also study the seroprevalence of Ballum, Copenhageni and Tarassovi in meat workers. Also farmers and veterinarians who have a close contact with these livestock species are at risk. With our findings at hand it is pertinent to investigate also their seroprevalence for these three serovars.

Ballum, Copenhageni and Tarassovi are serovars maintained by wildlife species (rodents, wild pigs, possums, hedgehogs), so it would be important to investigate occurrence of these serovars in wildlife species. Ballum is an important serovar with its high frequency in notified human cases. The frequency of Ballum ranges between 14.0% and 35.0% in the latest reports (Anon., 2011; 2012; 2013; 2014). Tarassovi is typically found in pigs; hence pigs are regarded as maintenance host for Tarassovi. Domestic pigs are kept by many farms in small numbers and there is an unknown rate of wild pigs with domestic livestock contact on pasture, and with humans during hunting. It would therefore be important to investigate the distribution of Tarassovi in domestic and wild pigs to identify trends over time in the distribution of Tarassovi in New Zealand.

Since the serovars are more common than expected it could be of value to explore the possibilities of including Ballum and Tarassovi in a vaccine. The challenge in that would be to convince the farmers to vaccinate their animals, since this would increase the cost of vaccination and even today, the majority of sheep, beef and deer farmers do not vaccinate their stock due to high cost relative to the potential benefits.

The prevalence of positive farms was high for all three serovars, there were farms where all animals were negative against one serovar. There were a lot more farms that were negative against Ballum and Tarassovi than against Copenhageni. This could depend on the distribution of serovars, but also be an effect of the fact that some of the farms vaccinated against Copenhageni. The results show that the prevalence in sheep herds is higher than in beef cattle and also mostly in deer. This could possibly imply that sheep are more likely to have higher contact rates with the wildlife species the serovars are adapted to. What more can be observed is that the prevalence for Ballum and Tarassovi is lower in deer herds than in beef cattle and sheep herds, which is similar to the results in prevalence seen at animal level. It can be speculated that deer interact with wildlife species in different ways than beef cattle and sheep.

There were only two deer farms from the Wairarapa region and both of them were free of Ballum and Copenhageni and one of them only had one positive animal against Tarassovi. From the Canterbury region there were 21 farms that had deer on their farm and the herd prevalence in this region was similar to the animal prevalence. The finding that the two negative farms only had deer might indicate that the deer are a less likely accidental host than beef cattle and sheep for these serovars. It would be interesting to investigate whether deer on multi-species farms are more likely to be exposed to different serovars than deer on only deer farms.

The equally high prevalence of all of the studied serovars in cattle and sheep may be explained by transmission across species due to grazing the same pasture at set stocking around calving/lambing. In view of the high prevalence, it may be hypothesised that sheep and cattle may be maintenance hosts, but more evidence is required to demonstrate a sustained prevalence in these species independent of external exposure.

Another topic to investigate is whether animals on one farm are more likely to be positive against one serovar if they already are positive against another serovar to see if there can be some association. However the kappa values from the overall prevalence indicates that this is unlikely, because no association could be seen at all in the overall prevalence between the different serovars.

Without taking species into account the prevalence of Ballum was lower in the South Island regions than in the North Island regions. It would probably be interesting to collect samples from the remaining regions on the North and South island to see if the prevalence was truly lower in the South Island. In the South Island, the prevalence of Ballum was also lower than the prevalence of Copenhageni and Tarassovi. This raises the question whether the wildlife distribution varies between the two islands. A possible explanation could be a higher prevalence of serovar Copenhageni and Tarassovi than Ballum among the wildlife species in the South Island. It would be interesting to have a look at the distribution of wildlife species occurring in areas around farms with a high prevalence of these serovars versus farms with a low prevalence.

Hedgehogs are maintenance host for Ballum but not for Copenhageni (Dreyfus et al., 2013) and it could therefore be of value to investigate the distribution of hedgehogs between the North and the South Island. If there for example would be a larger population of hedgehogs in the North Island it could possibly explain why the prevalence of Ballum is higher in the North Island.

In a study from 1981 it was hypothesized that a less well-adapted serovar could reach a higher prevalence in an area that is lacking a more highly adapted serovar to that specific ecosystem (Hathaway, 1981). This happened in Norwegian rat (rattus norvegicus, also named brown rat) populations in New Zealand on the southern half of the North Island. Norwegian rats are common maintenance hosts for the serovar Copenhageni, however in the absence of Copenhageni a high prevalence of Ballum was found in some Norway rat populations. During the time of this study this phenomenon had not been observed anywhere else and therefore it was hypothesized that this could only occur in absence of Copenhageni (Hathaway, 1981). These rats had been tested for the five serovars Ballum, Copenhageni, Hardjobovis, Pomona and Tarassovi (Hathaway, 1978). This could be a possible explanation to the difference in occurrence between serovars in different regions, as for example if the rat population on the North Island have a higher seroprevalence of Ballum than the rat population on the South Island, this could give rise to a higher risk of exposure to Ballum. If such an association existed however, there would be a negative association between serovar prevalence. Based on the extremely low Kappa values, our data suggested to the contrary that there was neither a positive nor a negative association between any of the three serovars in any of the three host species. Hathaway's (1981) observations could therefore be attributed to a difference in serovar-specific environmental risk factors (Heuer et al., 2015).

The confidence intervals in these results were calculated based on the assumption that the samples were completely independent. However this is not entirely true, because 20 samples come from each herd and 20, 40 or 60 samples from each farm, which makes them correlated to some extent. Accounting for the correlation would reduce the "effective" sample size and widen the confidence intervals, but the required adjustment was likely small as most farms were infected and prevalence was not strongly clustered. Another thing which has not been taken into account is the fact that the samples were sampled during different times of the year and the rate of infection can possibly vary between different seasons. The prevalence has not either been calculated accounting for the precision of the diagnostic test, which is not 100% sensitive and specific. The sensitivity and specificity can vary between different serovars and have not been calculated for Ballum, Copenhageni and Tarassovi in New Zealand.

When the prevalence was calculated for the different regions throughout New Zealand, it was made without considering the sampled animal species. Taranaki had a very high prevalence for all of the three serovars, however when looking at those results it has to be taken into account that there were only 40 samples and coming from a single farm. Many positives were found on this

farm giving this region, a possibly biased, relatively high prevalence. To get a more representative prevalence for the region more farms would have to be sampled. Similar considerations hold for Waikato.

Since the results were a lot higher than expected, it was important to exclude possible testing errors in the laboratory. The standards (the control plates that had been set up with antiserum of the requested serovar) used every day for the MATs were investigated and compared to other standards done months and years before. The standards are supposed to have similar end-point to each other. If one standard was positive at a higher dilution than previously, the tests done that day had to be correlated against the higher dilution in the standard, which would have to be taken into account when reading the plates. However the investigation did not show any apparent differences that could have affected the results. It was also investigated if there was any trend in the readings, e.g. if there were a lot more positives in the first days of reading than in the last days it could be due to the reader being unaccustomed to the reading in the beginning. However, no such trend could be observed. The cross-reactivity for the serovars was also tested, including available titres against Hardjobovis and Pomona, but no significant correlation between the serovars could be seen. No faults in the reading of the results could thus be identified and no cross-reactivity was seen, and we were therefore confident to rely on the results. One point of consideration, however, is that the samples had been stored for 6 years, although storage at -80 degrees is not likely to result in a titre increase, rather reduce titres or have no effect. It could however be interesting to evaluate how long-term storage of serum samples affects the MAT titres of leptospires.

The reason for setting the titre cut-off point to 1:48, which is low in an international comparison, is because we aimed to investigate previous exposure to *Leptospira* serovars rather than current infection or clinical illness. The latter would require a much higher titre cut-off. In some other countries the cut-off point is more usually set to 1:96, because when there are more serovars it can give rise to cross-reactivity at 1:24 and also at 1:48 (Dreyfus et al., 2013). However, because there are only six serovars, which are not too closely related, occurring in New Zealand it is not likely to get that kind of cross-reactivity (Collins-Emerson, personal communication). It is also important to set the cut-off point at 1:48 to be able to compare with other studies done in New Zealand (Dreyfus et al., 2013). However if the cut-off point should be set at 1:96 instead we would get a prevalence that is much lower than the one currently seen. Figure 2 clearly shows that there are few titres above 1:48 for Ballum and the titres for Tarassovi and Copenhageni are similar. This means that there are a lot of positive samples that are only positive up to 1:48, indicating previous exposure but perhaps less current infection. This is especially true for Tarassovi, where a lot of positive samples had the end-point at only 1:48.

6. CONCLUSION

The seroprevalence of Ballum, Copenhageni and Tarassovi was higher than in previous studies. This means a higher risk for the human health through more possible transmission routes of infection than first thought to be likely. It could explain the repeated findings of these serovars among notified human cases, in addition to the more common serovars Hardjobovis and Pomona. No association between the different serovars could be found in the overall prevalence. Our findings propose that more research is needed to investigate the transmission routes of these serovars between wild and domestic animal species. Future studies will show whether the serovars are likely to be common among wildlife species, which would suggest more research of *Leptospira* in wildlife of which there is little information available to date.

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APPENDIX

I. MAT protocol

Leptospirosis Research Unit
Infectious Diseases, IVABS
Massey University.

Species: Report results to: Date read:

Masterplate no.: Row: Antigen tested:

cohort	serum #	24	48	96	192	384	768	1536	3072	titre

Comments:

MAT read by:

II. **Master plate protocol:**

Leptospirosis Research Unit
Infectious Diseases, IVABS
Massey University.

Species: Farm: Submitted by:

Master plate #. Serum dilution: Date prepared:

A1											

Species: Farm: Submitted by:

Master plate #. Serum dilution: Date prepared:

A1											

III. Additional tables:

Additional table 1. *Table over number of animals from each region.*

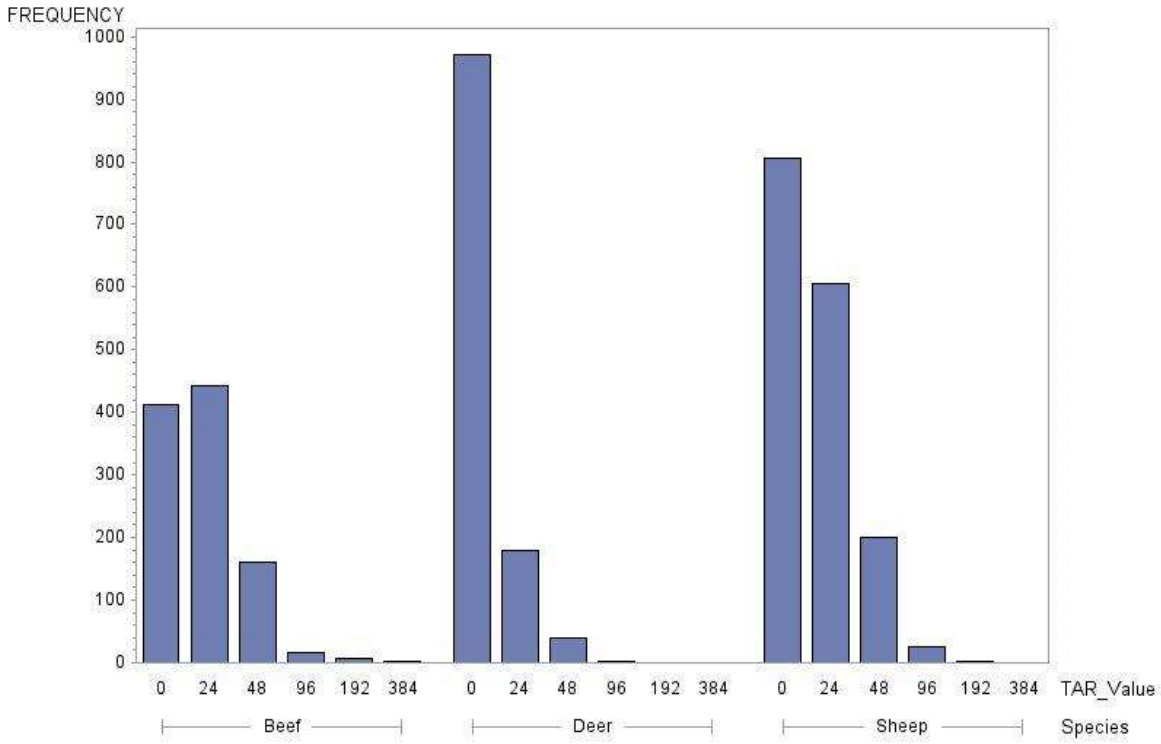
Region	Beef cattle	Deer	Sheep	Total
Canterbury	218	421	420	1059
Southland	53	188	200	441
Otago	40	80	199	319
Manawatu-Wanaganui	276	199	260	735
Hawke's Bay	179	140	220	539
East Coast	77	40	100	217
Waikato	60	65	63	188
Wairarapa	140	40	160	340
Taranaki	-	20	20	40

Additional table 2. *Seroprevalence in % of Leptospira serovar Ballum, Copenhageni and Tarassovi in different regions of New Zealand*

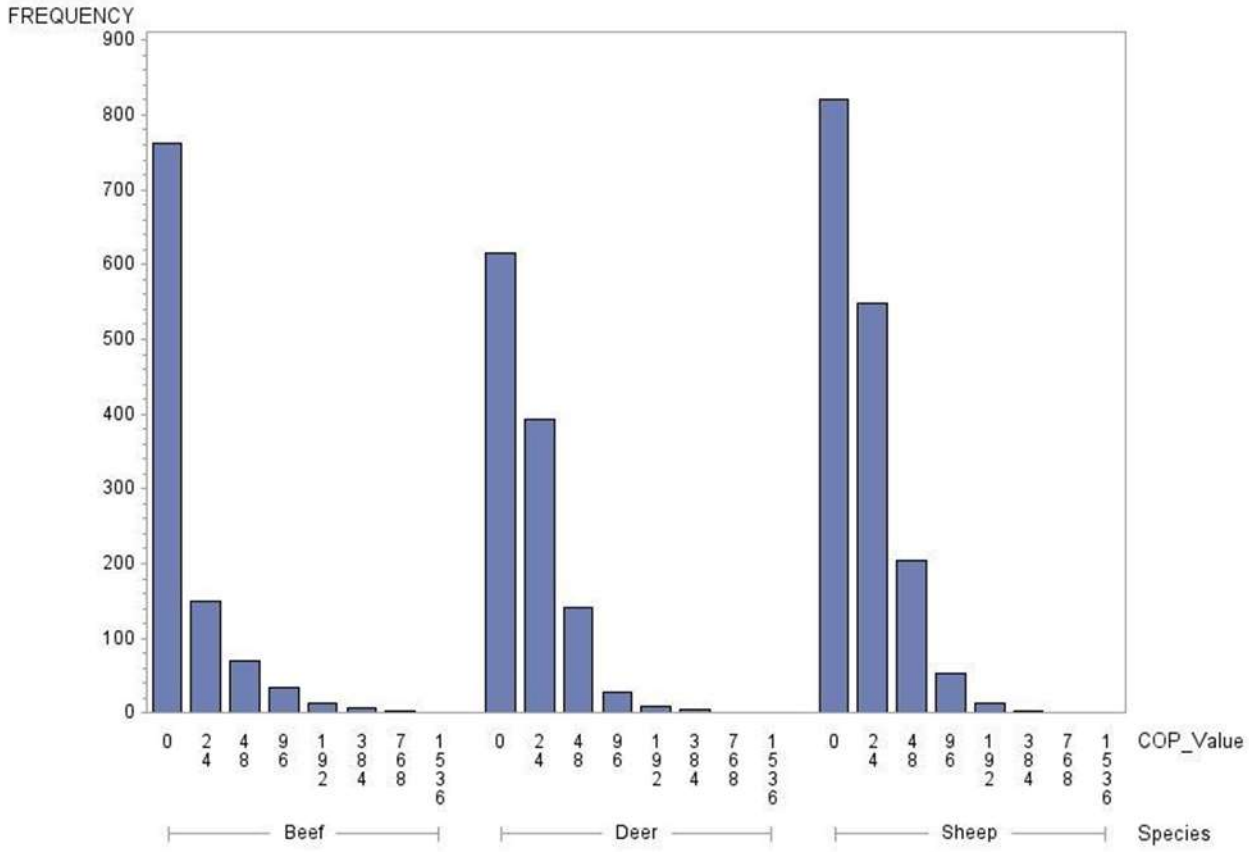
Region	Number of samples	Ballum	Copenhageni	Tarassovi
Canterbury	1059	8.2	10.3	7.2
Southland	441	9.8	16.1	15.0

Otago	319	9.1	13.2	17.2
Manawatu – Wanaganui	735	11.4	16.1	15.1
Hawkes bay	539	10.6	15.2	10.6
East coast	217	14.3	12.9	15.7
Waikato	188	9.6	38.8	13.3
Wairarapa	340	10.9	13.8	7.7
Taranaki	40	20.0	50.0	27.5

IV. Additional figures:



Additional Figure 1. *The distribution of samples according to the Tarassovi titre cut off.*



Additional Figure 2. *The distribution of samples according to the Copenhageni titre cut off.*